RESPIRATORY METABOLISM IN A MARINE DINOFLAGELLATE [1]

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The discovery by Sweeney and Hastings (1958) that cell division can be readily phased in *Gonyaulax* has increased interest in marine dinoflagellates for the analysis of metabolism in dividing cells. However, with the exception of some general data on photosynthesis in *Gonyaulax* (Hastings *et al.*, 1961) and *Gymnodinium* (Moshkina, 1961) as well as on the excretion of substances (Wangersky and Guillard, 1960), very little is known about the energy metabolism of dinoflagellates. And perhaps as unfortunate, even less can be assumed, for it has become apparent in recent years that the way in which energy metabolism is patterned in different algal groups can be quite diverse. We report here some experiments on the respiratory metabolism of intact cells of *Gymnodinium*, a common marine dinoflagellate.

MATERIALS AND METHODS

Pure cultures of *Gymnodinium nelsoni* (clone GSBL) were obtained from Dr. Guillard of the Woods Hole Oceanographic Institution. These were cultured in an enriched sea water medium (see Guillard and Ryther, 1962). Growth was determined, by mounting 1-ml. samples of cells immobilized with potassium iodide in a Sedgewick-Rafter cell. Growth was under either continuous light (400 foot candles) or in an 18-hour light- alternating with a 6-hour dark-period. In the latter case, cell division was somewhat phased, up to five times as many cells dividing per unit time within the dark-period than in the light-period. During the light-period, less than 2% of the cells could be seen dividing at any one time; during the 6-hour dark-period, 10% were dividing. The mean generation time for cultures at 21° C. was 3–4 days. Cultures were used for respiratory studies at densities between 7000 and 30,000 cells/ml., equivalent to about 150–600 μg. dry weight/ml.

In their studies of blue-green algae, Kratz and Myers (1955) found that cells suspended in phosphate buffer, bicarbonate buffer, or in the original growth medium all had equal respiratory rates. Our cells behaved similarly and we therefore adopted the third and simplest technique. Oxygen uptake was assayed with a Teflon-covered oxygen electrode (Kanwisher, 1959), with the platinum held −1.25 V. to the reference electrode, and 0.5 N KOH electrolyte. The electrode reaction itself consumes a molecule of O_2 for every 4e- of current flow. For

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small closed systems, this effect might produce a significant error; with large respirometers, it can be ignored.

Samples of cells were drawn aseptically from growing cultures and transferred into vials of 22-ml. size. A small stirring bar was placed into each respirometer. Each vial was then stoppered with a rubber stopper into which was fitted an O_2 electrode and a 16-gauge hypodermic needle (see Hochachka *et al.*, 1962). In operation, the vials were held in a 21° C. regulated water bath and stirred magnetically. Substances and inhibitors were added in volumes 1/100th that of the respirometers through the hypodermic needle without interrupting stirring of the cell suspension. The suspension was centrifuged at the end of a run, the cells were washed with H_2O, then dried to constant weight for Q_{O_2} calculations.

Results

A typical Q_{O_2} (dry weight) for cells in exponential phase of growth under continuous light was about 4.4 mm.^{3} O_2/mg. hour. In cultures grown under alternating light- and dark-periods, the respiratory rate fluctuated. Rate figures for cells in the dark-period were as low as 1.5 mm.^{3} O_2/mg./hour; three hours before the onset of the dark period, the cells had a Q_{O_2} of about 2.5.

Most of the compounds tested, over concentration ranges of 10^{-4} to $10^{-2}M$, produced no change or less than a 5% change in endogenous respiration. These included: glucose, fructose, galactose, glucose-1-phosphate, glucose-6-phosphate, fructose-1,6-diphosphate, ribose, glyoxylate, glycocoll, serine, α-alanine, methionine, glutamine, cystine, oxidized glutathione, diphosphopyridine nucleotide (DPN), DPNH, Coenzyme A, EDTA, and diethyl dithiocarbamate (DEC).

Respiratory inhibition was obtained by the compounds listed in Table I. Variations from the averages given were usually less than 10%. The values are based on final rates after treatment. The concentrations given are those found maximally effective. Poisons of the glycolytic enzymes did not depress respiration as effectively as those of the Krebs cycle. Inhibition with malonate and fluoroacetate was rapid, a new respiratory rate being established within a few minutes after the poisons were applied to the cell suspension. This rate, once established, did not change with time. However, in cells grown in alternating light- and dark-periods, the effectiveness of these poisons varied. In Table II, malonate sensitivity is recorded as a function of the light-dark periods. The values again are based on final rates after malonate addition compared to control rates. Respiratory blockage was greatest at the beginning of the dark-period and in the middle of the light-period. Two alternative explanations for this finding are available: (1) changes in permeability or (2) changes related to cell division (Stern, 1959; Sorokin and Myers, 1957) which, it will be recalled, was phased, about 5 times as many cells dividing per unit time in the dark-period than in the light. The last alternative is unlikely, since the generation time of the culture was long enough (3.5 days) to yield at any one time populations with cells at all stages of maturation.

The inhibition of respiration produced by the addition of oxaloacetate, citrate, and α-ketoglutarate is not understood. Oxidation of dihydroxyacetone dimer,

TABLE I

The effect of various compounds upon the O_2 uptake of Gymnodinium, *recorded as per cent change from the endogenous rate*

Inhibitory compounds	Concentration	No. of tests	Per cent change
Iodoacetate	10^{-3} *M*	12	−10
Fluoride	2×10^{-3} *M*	2	0
Arsenite	3×10^{-3} *M*	3	−51
Malonate	10^{-3} *M*	7	−65
(succinate restoration)	6×10^{-2} *M*	7	15
Fluoroacetate	10^{-2} *M*	4	−87
Azide	10^{-3} *M*	2	−52
Cyanide	3×10^{-3} *M*	2	−35
Oxaloacetate	10^{-3} *M*	6	−75
Citrate	10^{-3} *M*	4	−65
α-ketoglutarate	10^{-3} *M*	3	−76
Stimulatory compounds			
Dihydroxyacetone dimer	2×10^{-2} *M*	4	34
Acetate	10^{-3} *M*	9	5
Succinate	10^{-3} *M*	10	15
Fumarate	10^{-3} *M*	4	5
Malate	10^{-3} *M*	4	5
Malate plus acetate, each at	10^{-3} *M*	4	5
β-alanine	2×10^{-2} *M*	2	18
Glutamate	2×10^{-2} *M*	2	21
Ascorbate	6×10^{-4} *M*	4	505
	1.2×10^{-3} *M*	1	600
	1.8×10^{-3} *M*	1	900
Reduced glutathione (GSH)	6×10^{-4} *M*	5	504
Cysteine	6×10^{-4} *M*	2	67

glutamate, α-alanine, acetate, fumarate, and malate presumably occurred *via* the Krebs cycle.

The striking stimulatory effect of ascorbic acid, reduced glutathione (GSH), and cysteine on the respiration of a marine alga has not been reported previously. Some typical examples of this effect are shown in Table I for cells growing under continuous light.

At physiological pH ascorbate does not normally autoxidize (Mapson, 1958).

TABLE II

Changing sensitivity of respiration to 10^{-3} M malonate during the light-dark cycle. Data expressed as per cent decrease from endogenous rates of O_2 uptake measured at the same time

Time in light period	No. of tests	% Decrease in O_2 uptake
6 hours	3	15
9	3	57
12	3	60
15	3	15
Time in dark period		
1–2 hours	3	94
3–4	5	31

In control respirometers containing only autoclaved sea water medium, we found no detectable autoxidation at low concentrations of ascorbate (10^{-5} to 10^{-4} M); at higher concentrations of ascorbate, a significant autoxidation occurred (Table III). So that corrections for autoxidation would be unnecessary in experiments with cell suspensions, concentrations of 10^{-4} M were used routinely. Over a concentration range of 10^{-5} to 10^{-2} M, cysteine and GSH did not autoxidize under our conditions.

TABLE III

The utilization of O_2 by autoxidation of ascorbate in an autoclaved sea water medium used for growing Gymnodinium, *expressed as mg. O_2 used per hour*

Test system	O_2 uptake
Sea water	0.01
Sea water + 6 × 10^{-4} M ascorbate	0.01
Sea water + 3 × 10^{-3} M ascorbate	0.04
Sea water + 5 × 10^{-2} M ascorbate	0.08

In other plants, a GSH-ascorbate electron transfer scheme is fairly well characterized (Mapson, 1958, for a recent review). Generally, arsenite and iodoacetate activate the oxidation of ascorbate to dehydroascorbate. DEC and cyanide inhibit ascorbic acid oxidase, but EDTA, GSH, and cysteine do not affect the purified enzyme from higher plants. The ascorbic acid oxidase is atypical in the slime molds in that cyanide and azide produce slight but definite activation (Ward, 1955).

With *Gymnodinium*, the compounds tested were found to affect ascorbate, GSH, and cysteine oxidations in a similar manner. The data are in Table IV.

In each case, several concentrations were tested. Only those at which active compounds were maximally effective are recorded in Table IV; the ranges of concentrations tested are indicated by compounds which did not seem to affect ascorbate oxidation. The effect produced by the active compounds was rapid and usually did not change over fairly short time periods.

As in higher plants, ascorbate oxidation in *Gymnodinium* is activated by arsenite but unaffected by iodoacetate. Cyanide enhanced ascorbate oxidation, as in the slime mold, but azide was without effect. Since the latter two compounds inhibited endogenous respiration, presumably by blocking the cytochrome chain, these findings rule out the possibility that ascorbate oxidation was catalyzed by cytochrome oxidase (Webster and Frenkel, 1953). The oxidation of ascorbate was also atypical in that it was unaffected by DEC, presumably because of lack of penetration.

Although EDTA did not affect respiration of control cell suspensions, it completely inhibited ascorbate oxidation. Again, this is not typical of the oxidase in other sources, and can be explained in two ways: either the oxidation may be proceeding by copper catalysis (Mapson, 1958) and not by a specific ascorbic acid oxidase; or secondly, since copper is known to be released during reaction of ascorbic acid oxidase, EDTA-inhibition may arise by chelation of this copper with concomitant inactivation of the enzyme. The action of GSH and cysteine on ascorbate oxidation might be explained in a similar manner (Mapson, 1958). By comparison, oxidized glutathione, cystine, and methionine did not affect ascorbate oxidation.

Table IV

The effect of various compounds on ascorbate, GSH, and cysteine oxidation in Gymnodinium, *expressed as per cent change. Numbers in parentheses refer to number of tests. Cells grown in continuous light*

Compound	Concentration	Percentage effect on oxidation of 10^{-4} *M*		
		Ascorbate	GSH	Cysteine
Malonate	10^{-3} *M*	−100 (3)	−100 (2)	−100 (2)
Fluoroacetate	10^{-3} *M*	−100 (3)	−100 (2)	−100 (2)
Iodoacetate	5×10^{-4} to 4×10^{-3} *M*	0 (5)		
Arsenite	2×10^{-3} *M*	+170 (3)		0 (2)
Azide	5×10^{-4} to 10^{-3} *M*	0 (3)	0 (2)	0 (2)
Cyanide	10^{-3} *M*	+ 40 (3)		
DEC	5×10^{-4} to 4×10^{-3} *M*	0 (3)		
EDTA	5×10^{-4} *M*	−100 (5)		
Cysteine	10^{-3} *M*	− 67 (2)		
GSH	10^{-3} *M*	− 75 (2)		− 94 (2)
Cystine	5×10^{-4} to 10^{-2} *M*	0 (2)	0 (2)	0 (2)
GSSG	5×10^{-4} to 10^{-2} *M*	0 (2)	0 (2)	
Methionine	5×10^{-4} to 10^{-2} *M*	0 (2)	0 (1)	0 (1)

The inhibition of the GSH-ascorbate path by malonate and fluoroacetate was also very pronounced, suggesting a coupling of such a chain to the Krebs cycle. This suggestion is in accord with Young and Conn (1956), who found that GSH oxidation occurred in mitochondria in the presence of catalytic amounts of ascorbic acid; reduction of oxidized glutathione was coupled to the oxidation of Krebs cycle intermediates. Though in *Gymnodinium* ascorbate oxidation seemed to be concentration-dependent (Table I), this likely was not due to a greater utilization of ascorbate *per se*. By calculation it can be shown that complete oxidation of 5×10^{-4} *M* ascorbate would deplete all available oxygen in our respirometers. As Figure 1 shows, this did not occur. Perhaps the concentration dependence of ascorbate oxidation is somehow related to a reaction-inactivation which we observed. A typical recording (Fig. 1) shows the pattern of decay in the ascorbate-stimulated respiration. This effect, which is also found in higher plants, may be due to lowered O_2 tensions, or to H_2O_2, which is thought to produce the reaction inactivation in higher plants (Tokuyama and Dawson, 1962).

A shortage of time prevented any but brief analysis of GSH and cysteine oxidation. On the basis of the data in Table IV and the fairly well characterized

system in higher plants, it is assumed that GSH oxidation is tied up with ascorbate metabolism. This, however, is in need of much further study. Perhaps it is worth mentioning that the reaction, cysteine + oxidized glutathione ⇌ GSH + cystine, can proceed spontaneously; hence, cysteine oxidation might simply represent GSH oxidation. Alternative explanations are available.

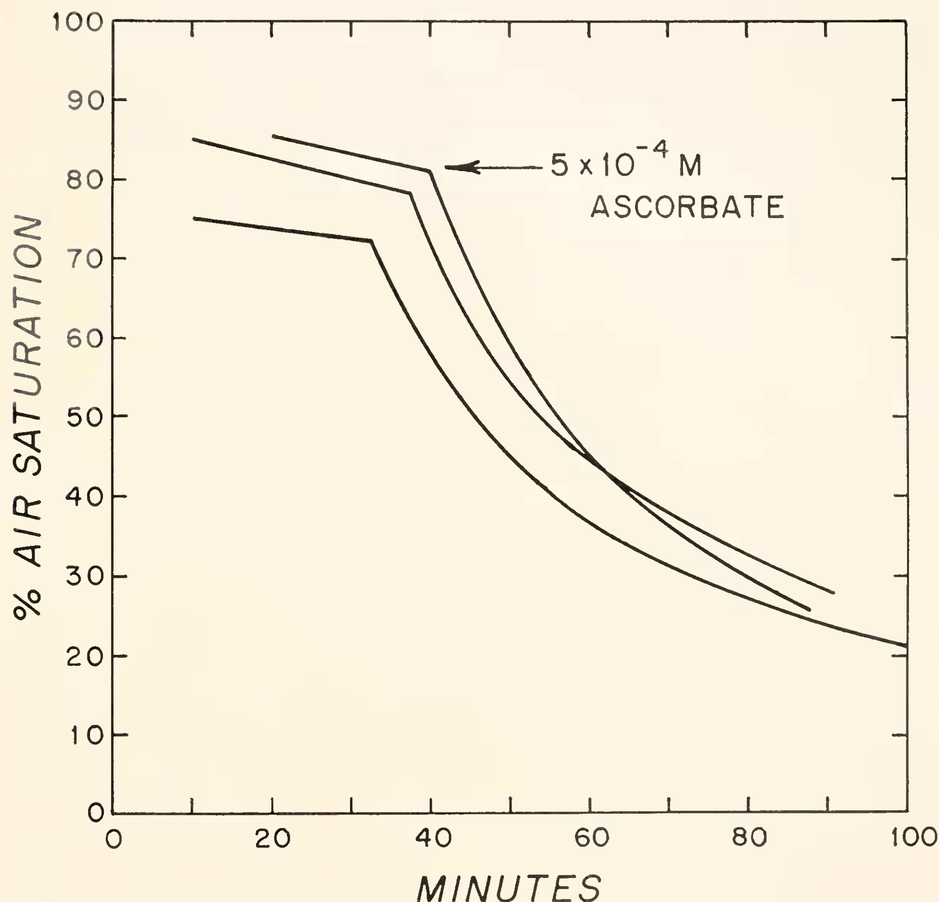

FIGURE 1. A typical recording of decreasing effect of ascorbate addition with time and oxygen concentration on metabolism of intact cells of *Gymnodinium nelsoni*. The experiment was done in triplicate. Rate of oxygen uptake is proportional to the slope of the lines.

From these pilot experiments, it is difficult to determine the contribution of a GSH-ascorbate system to the endogenous respiration of the organism. The large residual respiration remaining after azide and cyanide treatments of normal cells presumably occurs *via* an electron transport chain other than the cytochromes. Evidence of such a situation exists for other algae: *Anabaena*, for example, is insensitive to CO and sensitive to cyanide and azide only at high concentrations (Webster and Frenkel, 1953); cytochrome oxidase apparently is absent in some

strains of *Chlorella* (Kolesnikov and Einor, 1961). However, if a portion of endogenous respiration in *Gymnodinium* depended upon a GSH-ascorbate electron transfer chain, one would expect an inhibitor to lower the endogenous rate and activating compounds to raise it. As Table IV shows, EDTA is an effective inhibitor while arsenite is an effective stimulator of ascorbate oxidation in these cells. But, as previously mentioned, EDTA has no effect on endogenous respiration and arsenite actually inhibits it. Therefore, there can be little if any participation of a GSH-ascorbate system in endogenous respiration of these algae. In higher plants, too, the full potential effect of ascorbic acid oxidase is not realized *in vivo*. In pea stem internodes, for instance, the enzyme is capable of consuming O_2 at a rate 40 times as great as the total respiration of the tissue (see Mapson, 1958). In algae, then, the physiological role of this system remains unclear.

Summary

1. The effects of various inhibitors and substrates on the respiration of suspensions of *Gymnodinium* were tested. Iodoacetate, arsenite, malonate, fluoroacetate, azide and cyanide all inhibited the endogenous rate to some extent. Malonate and fluoroacetate were most potent. Cells grown in cycles of alternating light and dark were most sensitive to malonate during the middle of the light-period and at the beginning of the dark-period. Most of the glycolytic intermediates used did not affect the endogenous respiratory rate. Krebs cycle intermediates either increased the respiratory rate (succinate, malate, fumarate) or markedly decreased it (oxaloacetate, citrate, α-ketoglutarate). With the exception of an increased O_2 consumption in the presence of glutamate, alanine, and cysteine, the O_2 uptake of *Gymnodinium* was unaffected by the amino acids tested.

2. Ascorbate and reduced glutathione increased O_2 uptake profoundly. This uptake was further stimulated by cyanide and arsenite; it was insensitive to diethyldithiocarbamate and azide; it was completely blocked by cysteine, EDTA, malonate and fluoroacetate.

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